# APPENDIX A:

**Carbohydrate-based microparticles as adjuvant for allergy vaccines: CBP coupled recombinant grass pollen allergen ("G-antigen") - a vaccine against grass pollen allergy**

To study whether carbohydrate-based particles ("CBP") can be used as adjuvant in a vaccine against grass pollen allergy, a previously described recombinant hybrid molecule ("G-antigen") consisting of the major grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6 (1) was coupled to carbohydrate-based microparticles and formulated to a vaccine ("G-CBP". Balb/c mice were immunized with the vaccine and development of grass pollen allergen specific IgG antibodies and T-cell response upon immunization were monitored.

## Coupling of the G-antigen to Sepharose beads (CBP):

The CBP used for the study were cross-linked agarose beads, pre-activated with N-hydroxysuccinimide to react with primary amino groups from the protein by forming a stable covalent bond. In this document the designation of "CBP" is used for these pre-activated beads.

The process for coupling of the G-antigen to CBP and preparation of a bulk solution of G-CBP included the following steps:

1. Preconditioning of the CBPs: 10 ml of the CBP suspension (in 100% Isopropanol, as delivered) were washed with 1mM HCl.
2. Preparation of the antigen to be coupled: An antigen solution containing 0,5 mg/ml G-antigen in 20mM $NaHCO_3$, pH 9.0 was prepared.
3. Coupling of the G-antigen to CBPs: The HCl washed CBP suspension was mixed with 20ml antigen solution and incubated for 2 hours at room temperature (20-24°C). After 2 hours the supernatant was removed.
4. Blocking of residual binding sites: The CBP-antigen conjugate was incubated with blocking buffer (0.1M Tris-HCl, pH 8.5) for 2 hours at room temperature (20-24°C). After 2 hours the blocking supernatant was removed.
5. Washing of the CBPs to remove non-coupled antigen: The CBP-antigen conjugate was washed in alternating cycles of 0.1M Tris-HCl, pH8.5 and 0.1M acetate buffer, 0.5M NaCl pH 4.5.

6. Preparation of a bulk suspension of G-CBP: The CBP-antigen conjugate was washed for several times with sterile 0.9% NaCl. Finally, sterile 0.9% NaCl was added to the CBP-antigen conjugate to a total volume of 20 ml.
7. Filling and storage: Aliquots of the bulk suspension in 0.9% NaCl were transferred into sterile 2 mL polypropylene cryo-vials (Greiner, Cat. No. 122 279) closed with a screw-cap and stored at ≤ 8°C.

Steps 1, 5, and 6 of the coupling process are carried out in a 500ml glass filter funnel having a nominal pore size of 1.0-1.6µm and yield a mean particle diameter of 30 µm. Exchange of buffers/solutions and separation of buffers/ solutions from the CBPs is done by application of suction via a vacuum pump. Steps 3 and 4 of the coupling procedure are carried out in sterile cell culture flasks on a shaker.

**Formulation of a G-CBP vaccine containing 0.1 mg G-antigen/ml:**

The amount of G-antigen coupled on G-CBP was determined by measuring the protein concentration in the bulk conjugate suspension using the QuantiPro BCA Assay Kit (Sigma Aldrich). The assay was performed according to the manufacturer's instructions leading to the following result:

G-CBP 0.9 mg G-antigen per ml bulk conjugate suspension

The G-CBP vaccine was generated by diluting 1ml of the G-CBP bulk conjugate suspension with sterile 0.9% NaCl to a total antigen concentration of 0.1mg G-allergen /ml. The vaccine was filled into 1.5mL silanized glass-vials and stored at 4°C.

**Immunization of mice:**

Immunization protocol: Groups of BALB/c mice (n=5) were immunized subcutaneously with the G-CBP vaccine. Immunization was done with doses containing 10µg G-antigen per mouse on days 1 and 28, or with CBP in PBS as a control. Blood samples were taken on day 0 (preimmune serum), day 27 (IS1), and day 55 (IS2). All mice were sacrificed and splenocytes were isolated for further investigation.

The CBP-coupled G-antigen induces an antigen specific IgG1 antibody response in BALB/c mice.

In order to study the development of a grass pollen allergen- specific antibody response upon immunization with the CBP-coupled G-antigen, blood samples were taken before and after immunization according to the immunization protocol and analyzed for G-antigen-specific

IgG1 antibodies by ELISA as described previously (2). All mice, which were immunized with the CBP-coupled hybrid molecule, developed a hybrid-specific IgG1 response, which could not be detected in the control group immunized with CBP in PBS.

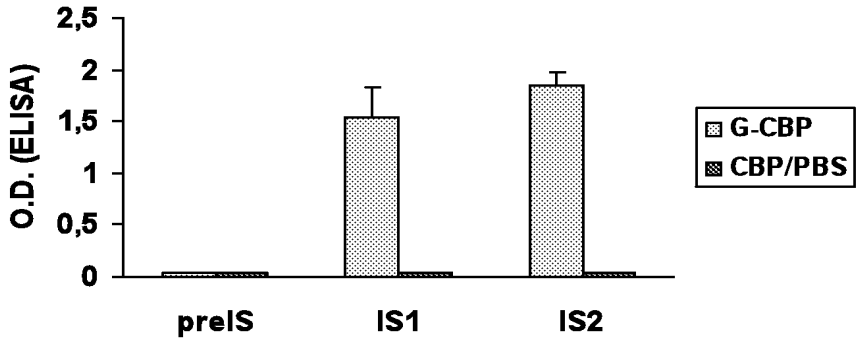


**Figure 1.** Development of a hybrid-specific antibody response in BALB/c mice using CBP as an adjuvant. Mean serum levels of hybrid-specific IgG1 antibodies of each mouse group measured by ELISA (O.D. y-axis) are shown.

G-CBP induces hybrid-specific T cells in BALB/c mice upon immunization.

In order to demonstrate that the CBP-adjuvanted G-antigen induces an antigen-specific T cell response upon vaccination, splenocytes from BALB/c mice, which had been immunized with G-CBP, were isolated and incubated with the antigen. Proliferation of cells was measured as previously described (3) and compared to a group of mice that had been immunized with CBP in PBS alone. We found that vaccination with CBP coupled G-antigen induced antigen specific cell proliferation, while the control group immunized with CBP in PBS did not.

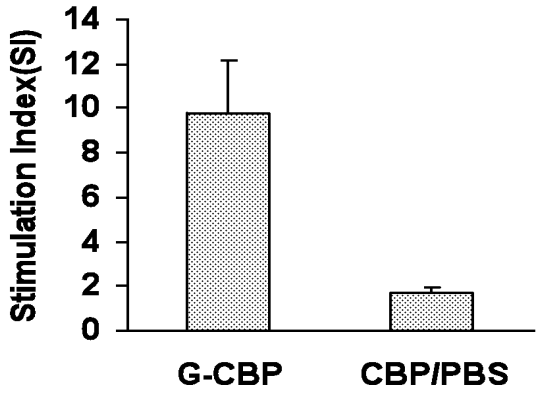


**Figure 2.** G-antigen-specific cell proliferation. Groups of mice were immunized with the G-CBP vaccine or CBP in PBS. Mouse splenocytes were isolated and stimulated with the G-antigen. Mean stimulation indices (y-axis) for each group are shown.

**References:**

1. Linhart B, Hartl A, Jahn-Schmid B, Verdino P, Keller W, Krauth MT, Valent P, Horak F, Wiedermann U, Thalhamer J, Ebner C, Kraft D, Valenta R. A hybrid molecule resembling the epitope spectrum of grass pollen for allergy vaccination. J Allergy Clin Immunol. 2005;115:1010-6.

2. Linhart B, Bigenzahn S, Hartl A, Lupinek C, Thalhamer J, Valenta R, Wekerle T. Costimulation blockade inhibits allergic sensitization but does not affect established allergy in a murine model of grass pollen allergy. J Immunol. 2007; 178:3924-31.